The Importance of Epithelial Uptake Systems in Lung Toxicity

by Lewis L. Smith,* Christian P. L. Lewis,* Ian Wyatt,* and Gerald M. Cohen†

The discovery that the herbicide paraquat was selectively accumulated by the lung, both *in vivo* and *in vitro*, in comparison with other tissues, provided an explanation for its selective toxicity to the lung. This uptake process is energy dependent and obeys saturation kinetics. A characterization of the process led to the identification of endogenous chemicals that are the natural substrates for the system. Among these are a series of diamines and polyamines, as well as the diaminodisulfide cystamine. It appears that paraquat, because of specific structural similarities to these endogenous polyamines, is mistakenly accumulated by the lung. This uptake process is specifically located in the alveolar Type II cell, the Clara cell, and probably the alveolar Type I cell.

With the development of knowledge of the structural requirements of chemicals to be accumulated by this system, it is possible to predict which chemicals will be accumulated by the lung or design molecules that are targeted to the alveolar epithelial and Clara cells.

In the wider perspective, this polyamine uptake system has been found on a number of cancerous cells or tissues. With the knowledge of the uptake system in the lung, it should be possible to design drugs that will be specifically concentrated in cells that possess this system.

Introduction

Because the lung is responsible for all gas exchange necessary for oxidative metabolism, it is inevitably exposed to toxic gases, vapors, and particles (if small enough) present in the atmosphere. The respiratory function of the lung requires a large surface area (in man, about the size of a tennis court) that, on the air side, is covered by epithelial cells intimately associated with an extensive capillary network. Apart from inhalation, the lung is also exposed to toxic compounds that have been absorbed into the bloodstream or are in the form of metabolites, formed either in the lung or in extrapulmonary tissue.

In order to meet its respiratory function, the lung requires numerous cell types. More than 40 cell types have been identified in the lung (*1*); these are required to provide the diverse architecture associated with cartilage, smooth muscle, connective tissue, submucosal glands, the vascular system, and the respiratory unit (alveoli) (*2*). Extensive literature describes the normal architecture of the lung and the interrlationships which occur between the numerous cell types (*2*).

The selective vulnerability of lung cells to toxins depends on a variety of factors. The route of administration of a toxicant, differences in the metabolism of a toxicant within individual cell types, or differences in the biochemical function of individual cell types may all contribute to rendering particular cell types susceptible to an individual chemical (*3*). In this review we intend to concentrate on the characterization of a particular uptake system in specific lung cells that is responsible for the accumulation of various endogenous and exogenous chemicals. Readers shall see that the presence of this uptake system allows specific epithelial cells to become a victim of selective damage, although the same system may also prove useful in targeting specific chemicals to individual cells for therapeutic purposes.

Background

In the last 25 years there have been a number of human fatalities as a consequence of the ingestion of the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridilium) (*4*). These fatalities have resulted largely as a consequence of the intentional ingestion of the concentrated commercial product. When it is swallowed, the symptoms of

*Biochemical Toxicology Section, ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK.

†Toxicology Unit, School of Pharmacy, Brunswick Square, London, WC1N 1AX.

Address reprint requests to L. L. Smith, Biochemical Toxicology Section, ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK.

poisoning depend largely on the amount consumed. Patients who die within a few days of its ingestion develop multiorgan failure associated with adrenal damage, liver damage, kidney damage, cerebral hemorrhage, and lung damage (*5*). In these cases the precise cause of death is difficult to establish since so many vital organs have been destroyed. However, patients who survive for many days or weeks after ingestion and then succumb to the toxin usually die directly or indirectly as a result of selective damage to the lung (*5*).

There are two distinct phases in the development of lung lesions provoked by paraquat (*6*). The first is a destructive phase in which the alveolar Type I and Type II epithelial cells are damaged within a few days of poisoning (*6*). If this damage is extensive, an alveolitis develops and is associated with frank hemorrhage, edema, and the infiltration of inflammatory cells into the interstitial and alveolar spaces of the lung. When paraquat is given to experimental animals (e.g., the rat), many will die within the first few days of dosing as a consequence of this alveolitis. Although there have been occasional reports of endothelial cell damage in the lung, it is generally accepted that the primary target cells of paraquat are those of the alveolar epithelium (*6*).

The second phase of the lung lesion is characterized by an extensive proliferative fibrosis (*6*). In experimental animals or humans who develop an extensive alveolitis but do not succumb during the first few days of poisoning, a fibrosis develops that can be so severe as to destroy the normal architecture of the lung and lead to death from anoxia (*6*). This fibrosis can be regarded as a consequence of the acute destructive phase and is probably part of the normal reparative response of the lung.

In one of the earliest experiments carried out to investigate the mechanism of paraquat toxicity in rats, it was found that after oral administration the plasma concentration of paraquat remained relatively constant over a period of 30 hr, whereas the concentration in the lung rose progressively to several times that in the plasma (*7*) (Fig. 1). In no other organ studied was this time-dependent accumulation of paraquat seen (*8*). This selective accumulation, in part, explained the selective toxicity of paraquat to the lung, since it was this organ that achieved the highest concentrations of paraquat after oral dosing (*8*). The only exception is the kidney that was found to contain high concentrations (*8*), since it is the route of excretion of paraquat. However, the disposition of paraquat in the kidney is a result of glomerular filtration, rather than a selective accumulation as seen in the lung.

In order to investigate the accumulation of paraquat into lung, Rose et al. (*9*) studied its uptake into rat lung slices. Using this method, they found that paraquat was accumulated in a time-dependent manner into slices by a process that was energy-dependent and obeyed saturation kinetics (*9*). Consequently, it is possible to derive an apparent K_m of 70 μM and a V_{max} of 300 nmole paraquat/g lung hr (*9*). The accumulation of paraquat into

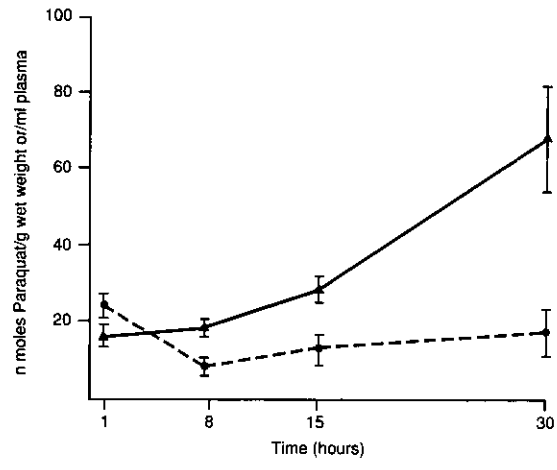

FIGURE 1. Rats were orally dosed with 680 μmole of paraquat/kg body wt and the lung (▲) and plasma (●) levels of paraquat determined. Each time point represents the mean ± SE for five rats.

tissue slices taken from various organs of the rat demonstrated that, apart from the lung, the brain cortex is the only other tissue able to accumulate paraquat (*8*). However, the uptake of paraquat into the brain only occurs *in vitro*, since there is no retention or accumulation of paraquat in the brain, *in vivo* (*8*). This difference is likely to be due to the blood-brain barrier that prevents accumulation *in vivo* but would not be relevant to *in vitro* studies. The discovery that paraquat was accumulated into the lung led to the search for endogenous compounds that are present in the plasma or lung and could be the natural substrate for the uptake system.

Uptake System Responsible for the Accumulation of Paraquat

In an attempt to identify possible endogenous compounds that could be the natural substrate for the uptake system in the lung, the ability of putative substrates to inhibit the uptake of paraquat into lung slices was investigated. A wide variety of compounds were tested, but among the most effective inhibitors of paraquat accumulation were a series of diamines and polyamines (Table 1) (*10*). Paraquat uptake is reduced in the presence of the diamine putrescine, and this reduction was dependent on the putrescine concentration (*11*). Putrescine itself was found to accumulate into lung slices in a linear, time-dependent manner by a process that is both energy dependent and obeys saturation kinetics (*11*). These observations led to the suggestion that there is competition between paraquat and putrescine for an uptake process in the lung. As with paraquat, it was possible to derive an apparent K_m for the accumulation of putrescine. This was found to be 7 μM (*11*) which is approximately 10-fold lower than that for paraquat, indicating that the endogenous substrate has a greater affinity for the uptake process than does paraquat. The

Table 1. The effect of diamines and polyamines on the accumulation of paraquat into rat lung slices.[a]

Compound	Structure	% of control
Cystamine	$NH_2(CH_2)_2S-S(CH_2)_2NH_2$	58
Putrescine	$NH_2(CH_2)_4NH_2$	31
Cadaverine	$NH_2(CH_2)_5NH_2$	65
Hexamethylenediamine	$NH_2(CH_2)_6NH_2$	29
Decamethylenediamine	$NH_2(CH_2)_{10}NH_2$	19
Spermidine	$NH_2(CH_2)_4NH(CH_2)_3NH_2$	71
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	54

[a]Slices of rat lung were incubated in Krebs-Ringer phosphate (KRP) glucose medium (37 °C) containing 10 μM [^{14}C]paraquat together with 10 μM of test compound. The accumulation of paraquat was monitored over 90 min, the linear rate of paraquat uptake determined, and the results expressed as the percentage of the control rate, which was 33 ± nmole paraquat/g wet wt/hr.

V_{max} for the uptake of paraquat and putrescine were almost identical, suggesting that there is a single system responsible for the accumulation of both compounds (*11*).

In addition to putrescine, a series of diamines and polyamines were found to use this uptake system (*12*). This leads to the conclusion that a polyamine uptake system is present in the lung that is capable of accumulating paraquat, such that when paraquat is absorbed into the bloodstream it circulates to the lung and is mistakenly accumulated for the natural substrates, the polyamines (*11*). Since, as stated previously, there are more than 40 different cell types in the lung, it was important to identify which cell (or cells) was responsible for this uptake process.

Location of the Uptake System in the Lung

The observation that paraquat selectively damaged the alveolar Type I and Type II epithelial cells and the Clara cells of the lung provided an initial hypothesis that these cell types may well be the compartments into which paraquat is accumulated. The first report to use autoradiographic techniques to visualize the compartment indicated that tritiated paraquat was confined almost entirely to cells having the typical distribution of alveolar Type II cells (*13*). Recently, it has been shown that in lung slices incubated with tritiated putrescine, spermidine, or spermine the label is concentrated in Clara cells, whereas adjacent ciliated cells remain almost entirely unlabeled (*14,15*). The alveolar Type II cells are also specifically labeled and there is diffuse label on or near the alveolar Type I epithelial cells. It is not possible with the resolution of the light microscope to be categoric that this cell type has accumulated the polyamines (*14,15*). However, since the Type I cell is damaged with paraquat and there is an absence of labeling in the vascular endothelium lining the larger blood vessels, it seems reasonable to conclude this cell also accumulates putrescine and, by analogy, paraquat (*15*).

Differences are seen between the use of lung slices *in vitro*, and perfused lung and lung *in vivo* studies. With the perfused lung and the lung *in vivo*, labeled putrescine, spermidine, and spermine were only found in the alveolar Type II cell; no label was seen either in the Clara cell or over areas of the alveolus associated with the Type I cell (*14*). This may reflect a real difference in the distribution of paraquat and the polyamines between *in vitro* and *in vivo* systems (*14*). However, the authors concluded that the difference is more likely to be attributable to differences in the specific activity of the labeled compounds that can be given *in vitro* compared with *in vivo* (*14*). With the lung slice technique, it is possible to expose lung cells to labeled compound of a high specific activity, whereas with both the perfused and *in vivo* lung, this is not the case (*14*).

In conclusion, the polyamines are accumulated into perfused lung and into the lung *in vivo*, as well as into lung slices *in vitro*. This accumulation certainly occurs in the alveolar Type II cell, the Clara cell, and very probably the alveolar Type I cell. By analogy, it can be argued that paraquat is also accumulated into these cell types.

Other Substrates for the Polyamine Uptake System

Studies in this laboratory have shown that the physiologically occurring thiol cysteamine is able to inhibit the uptake of paraquat into lung slices (*16*). However, it is known that in plasma or extracellular fluid, cysteamine will at least, in part, be oxidized to the disulfide cystamine (*16*). Moreover, since cysteamine is a monoamine that is known not to use the transport system, it seemed likely that cystamine, which does meet the criteria for uptake via the polyamine system, may itself be accumulated. This is indeed the case (*16,17*). Cysteamine is not able to inhibit the uptake of paraquat into lung slices if the cysteamine sulfhydryl group is stabilized by the addition of dithiothreitol (DTT) to the incubation medium. When cysteamine undergoes autooxidation in the medium to cystamine, it is able to inhibit paraquat uptake (*16*). Cystamine accumulation into lung has been directly measured (*16,17*) and it appears to result from two separate processes with different kinetic parameters (*16,17*). A high-affinity system has been identified, and this appears to be responsible for the uptake of polyamines (*16,17*). The system obeys saturation kinetics and is energy dependent (*16,17*). The identification of a cystamine uptake system into the lung raises the question as to whether this, or the polyamines, or both are primary substrates for the uptake process. Since the polyamines were the first endogenous substrates identified to use this process, the system has become known as a polyamine uptake system in the lung. However, there are compelling reasons to suggest that cystamine is the prime substrate for the uptake process in the alveolar epithelial cells. On accumulation into the lung, cystamine is rapidly metabolized to taurine (*16,17*). This is the major metabolite for both the high-affinity and low-affinity systems (*16,17*).

The taurine that is formed in the lung is wholly retained as taurine, suggesting an endogenous function for taurine in the lung (*16*). Taurine is found in high concentrations in several tissues that generate reactive oxygen species, and among its other effects it is known to possess antioxidant properties (*18*). Moreover, several disulfides including cystamine have been suggested to have the potential to regulate cellular NADPH levels in response to oxidative stress (*19*). Since the apparent K_m for the pulmonary uptake of cystamine (12 μM) is similar to the plasma concentration of the disulfide (*20*), it seems plausible to suggest that *in vitro* this uptake system is, in part, responsible for the accumulation of cystamine and, consequently, the presence of taurine in the lung. It is plausible to suggest that both compounds act as antioxidants in the lung. This would be consistent with the known requirement of the lung to maintain, at its epithelial cell surface, an adequate defense against the oxidative stress caused by the continuous exposure to high concentrations of oxygen.

Targeting of Radioprotective Agents to the Lung

Since the structural requirement for chemicals to act as substrates for the polyamine uptake system has been investigated (*21*), it is possible to predict those structures that are likely to have the requirements for accumulation by the lung. The more effective substrates are those with at least four methylene groups between quaternary nitrogen atoms (putrescine, cadaverine, etc.). Their maximum inhibitory potency was found when there were between 7 and 10 methylene groups (*10*). So far, it has not been possible to establish the optimum separation between the nitrogen atoms. However, the minimum separation appears to be in excess of 0.5 nm (*22*). We have found that the radioprotective compound WR2721 [*S*-2(3-aminopropylamino)ethyl phosphorothioate] can act as a substrate for the uptake system (*23*). However, the separation of the nitrogen atoms in WR2721 by three methylene groups suggested that this molecule would be a relatively poor substrate.

The ability of a series of *S*-2(aminoalkylamino)ethyl phosphorothioates to competitively inhibit the uptake of putrescine into the lung has been measured and used as an indirect measure of the ability of these radioprotectors themselves to be accumulated into the lung (*23*). The substrates used were WR2721 and *S*-2(4-aminobutylamino)ethyl phosphorothioate (*S*-ABEP) and *S*-2(7-aminoheptylamino)ethyl phosphorothioate (*S*-AHEP) (*23*). Their inhibitory potency tended to increase as the number of methylene groups between the nitrogen atoms increased (Table 2). The apparent K_m for putrescine accumulation into lung slices is approximately 15 μM whereas the K_i values determined for WR2721, *S*-ABEP, and *S*-AHEP were 48, 57, and 7 μM, respectively (*23*). Since the inhibition of putrescine accumulation by these phosphorothioates was competitive (Fig. 2), it can be argued that the apparent K_m for the uptake of these chemicals would be 48, 57, and 7 μM, respectively.

Table 2. The effect of the *S*-2-(aminoalkylamino)ethyl phosphorothioate radioprotectors on the accumulation of putrescine into lung slices.[a]

Compound	Structure	K_i, μM
WR2721	$NH_2(CH_2)_3NH(CH_2)_2$ S	48
S-2-(3-Aminopropylamino) ethyl phosphorothioate	O=P—OH OH	48
S-ABEP	$NH_2(CH_2)_4NH(CH_2)_2$ S	
S-2-(4-Aminobutylamino) ethyl phosphorothioate	O=P—OH OH	57
S-2-(7-Aminopeptylamino)	$NH_2(CH_2)_7NH(CH_2)_2$ S	
S-2-(7-Aminopeptylamino) ethyl phosphorothioate	O=P—OH OH	7

[a]The inhibitory constants were derived from the Lineweaver-Burk plots of [^{14}C]putrescine uptake into lung slices in the presence of 100 μM WR2721, 50 μM *S*-ABEP, or 10 μM *S*-AHEP. The inhibitory constants were determined using the method of Dixon and Webb for competitive inhibition. The linear regression correlation coefficient for all plots was greater than 0.9.

As stated previously, we have shown that cysteamine is rapidly converted to the disulfide cystamine under the conditions for investigating its accumulation into lung slices. The oxidation of cysteamine and, consequently, its ability to compete for the uptake site, is prevented if DTT is present in the incubation medium, since this maintains the cysteamine as a monoamine containing a sulfhydryl group (*16*). In the absence of DTT the cysteamine is oxidized to cystamine (*16*). Since WR2721 is rapidly dephosphorylated by alkaline phosphatase to form the free thiol *N*-2-mercaptoethyl-1,3-diaminopropane (*24*), by analogy with cysteamine these dephosphorylated radioprotectors may be oxidized to the disulfide in the absence of DTT. That this is the case can be demonstrated by the effect of DTT on the ability of WR2721 to inhibit putrescine accumulation into the lung. The apparent K_i was increased from 48 μM in the absence of DTT to 155 μM in its presence, indicating that as the sulfhydryl WR2721 was a less adequate substrate for the uptake system (*23*). Similarly, the K_i of *S*-ABEP was increased from 57 to 88 μM and the K_i of *S*-AHEP from 7 μM to 15 μM (*23*). These results are indicative that the free thiols are spontaneously oxidized to the disulfide in the incubation medium, and that these radioprotectors in this form are more effective at competing for the uptake site. This suggests that the transport receptor in the lung recognizes the diamine structure in either its free thiol or disulfide form, although chemicals act as more effective substrates in a disulfide form probably as a result of the greater separation between the nitrogens.

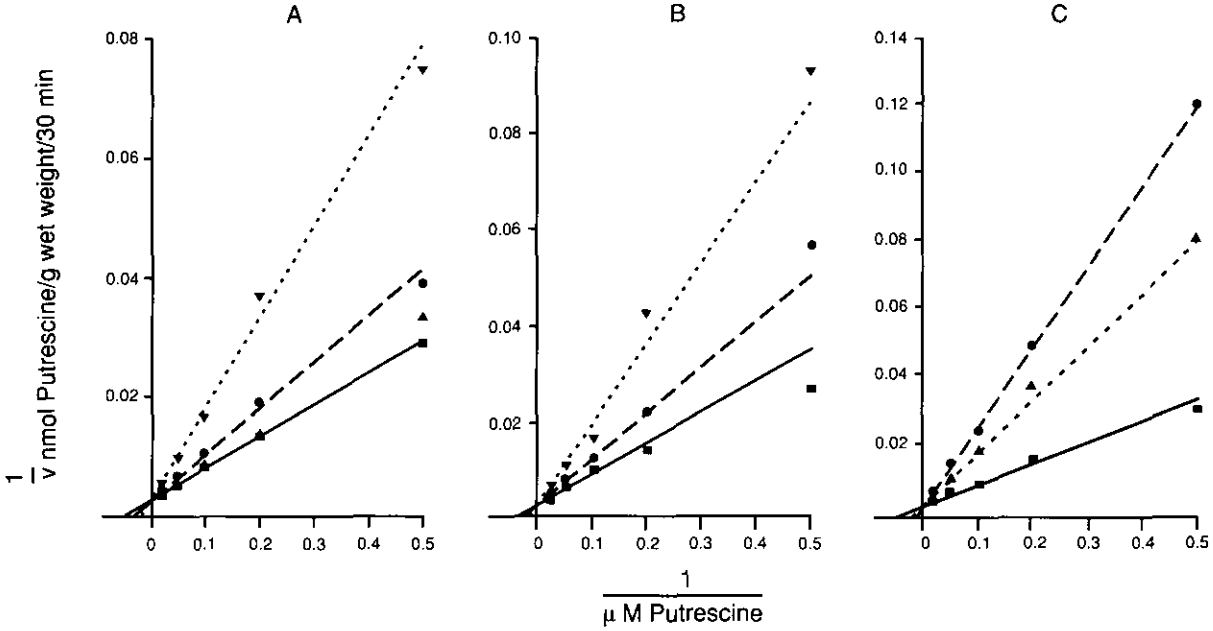

FIGURE 2. The kinetic profile for the uptake of [^{14}C]putrescine into lung slices in the presence of (*A*) WR2721, (*B*) S-ABEP, and (*C*) *S*-AHEP. (■-■-■) control, (▲-▲-▲) 10 μM, (●-●-●) 25 μM, and (▼-▼-▼) 100 μM depicted by Lineweaver-Burk plots. Each point was the mean of three observations.

These results, together with a greater understanding of the structural requirements for substrates to use this uptake system, suggest it may be possible to target a wide variety of chemicals to the alveolar Type I and Type II cells and Clara cells of the lung. It is clear that by altering the number of methylene groups separating the amine groups in the substrate, it is possible to alter the affinity of the chemical for the uptake process. It seems reasonable to argue that *S*-ABEP and *S*-AHEP may be more effectively accumulated by the lung than WR2721, thereby providing a more targeted and, consequently, more affective radioprotection (*23*).

However, a feature of the kinetics associated with this transport process indicates that there are differences between *in vitro* and *in vivo* studies. We know that in the case of paraquat, following oral dosing to rats, it takes many hours of relatively constant concentrations in the plasma to achieve high concentrations of paraquat in the lung (*7*). Similarly with putrescine, it is many hours before the lung accumulates concentrations in excess of those found in the plasma (*14*). The reasons for the difference between *in vivo* and *in vitro* kinetics are not fully understood. They are likely to include the presence of other endogenous substrates in the plasma *in vivo*, compared with *in vitro* studies, and the likelihood that the exposure of the substrate to the lung epithelial cells *in vitro* is considerably greater than that following *in vivo* dosing in which the substrate is delivered to the lung via the endothelial capillary network. This means that to optimize the targeting of radioprotectors to the lung *in vivo*, it may be necessary to administer the compounds so that they are present in the plasma for many hours and so that the concentration is in the range of the apparent K_m for the transport process. This suggestion is in contrast to the current regime for the administration of WR2721 to patients prior to radiation or chemotherapy treatment for malignancy (*24*). Moreover, if the results from *in vitro* studies are relevant to the *in vivo* situation, then WR2721 does not have the optimum molecular structure to use the polyamine transport process; *S*-ABEP and *S*-AHEP have a higher affinity for the transport system than WR2721.

General Perspective

An examination of the wide range of chemicals that can act as pulmonary toxicants reveals the phenomenon of selective toxicity to individual cell types in the lung. This selective toxicity depends on a number of factors such as the route of administration of the toxicant, its disposition, metabolism, and the specific biochemical and physiological characteristics of the individual cell types where the toxicant is targeted. In this brief article I have attempted to highlight the role of a polyamine accumulation system in specific epithelial cells of the lung in determining the selective toxicity of the herbicide paraquat. It is the concentration of this toxicant in specific lung cells that leads to selective damage to these cells and, consequently, the observable selective

toxicity in pulmonary tissue. Perhaps more important than the role of the system in leading to selective toxicity is the possibility that it can be exploited for the delivery of selected molecules to individual epithelial cells in the lung. So far, only the radioprotectors WR2721 and its analogues have been investigated, but from the structural requirements for this system, it is possible to predict a wide range of substrates that are likely to use this up take system.

In the wider perspective, the lung is not the only organ with the ability to accumulate polyamines. Polyamine accumulation has been described in the brain (*12*), salivary glands (*25*), seminal vesicles (*25*), nontransformed AD3 white cells (*25*), murine leukemic WEHI3 white cells (*25*), prostatic cancer cells (*26*), neuroblastoma cells (*27*), and ascites L1210 leukemic cells (*28*). It appears that the system described in those various cell types is identical or very similar to that which has been described in the lung. Theoretically, it should be possible to target a diverse range of substrates that meet the structural requirements for the polyamine uptake system to these individual cell types, offering the possibility of designing cytotoxic drugs that will be selectively accumulated by cancer cell types that possess the polyamine uptake system.

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